FURTHER INVESTIGATIONS ON NITROGEN-FIXING BACTERIA IN SOIL.

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Introduction.

In an earlier paper (Swaby, 1939) it was shown that waterlogging a soil tends to increase the numbers of *Clostridium butyricum*, and it was suggested that anaerobic nitrogen fixation might occur after heavy rains. It was also found (Jensen, 1940) that considerable amounts of nitrogen may be fixed in waterlogged soil, but apparently chiefly through the activity of *Azotobacter*. The following investigations were designed to test, firstly, how far the development of *Azotobacter* and *Cl. butyricum* could be affected by varying the water content of the soil between a comparatively moist state and saturation point, and, secondly, whether a correlation could be traced between multiplication of *Cl. butyricum* and nitrogen content of the soil. In addition, some supplementary data on the distribution of *Azotobacter* and the possible existence of other nitrogen-fixing bacteria in Australian soils are presented.

1. *Influence of Soil Moisture on Nitrogen-fixing Bacteria.*

The soil used for this investigation was taken from a plot in the Melbourne University grounds: immature grey sandy-loam, of pH 7·2, and containing 0·10% total-N and 1·73% organic carbon.† The plot has been used for growing cereals for a period of years, and has received heavy applications of lime and superphosphate, in recent years also a small quantity of manganese sulphate.

Schofield (1935) pointed out that the air-water relationship of a soil could not be measured by moisture determination alone, and suggested the use of the term pF, which is the logarithm of the capillary tension; this is used as an index of the aeration and water content of the soil in the present study. The filter paper method (Gartner, 1937) was found to give good results up to the sticky point (18% H_2O).

Bacterial counts were made by the methods previously described (Swaby, 1939). Each count was triplicate, i.e., a minimum difference of 80% between counts is necessary for significance.

An experiment was first designed to imitate field conditions after light and heavy rains and their influence on N-fixing bacteria. A bulk sample of soil was collected at the end of the summer, and 4 sub-samples, of approximately 150 gm., were made up to 6, 16, 28 and 38% moisture. Replicate sub-samples were incubated at 22°C. in Petri dishes of 10 cm. diameter, and loss of weight by evaporation was made up periodically by addition of water. Counts of *Azotobacter* and *Cl. butyricum* were made prior to incubation and subsequently at intervals of a few days by removing several cores of soil from each Petri dish and mixing together to form 4 composite samples. The results of these counts are given in Table 1.

* The first section of this work was carried out by the junior author in the Department of Bacteriology, University of Melbourne. The third section was carried out by the senior author, and the second section by both authors in collaboration.

† This abnormally wide C/N ratio was doubtless due to the presence of some material from asphalt road sweepings.

TABLE 1.
Effect of Soil Moisture Content on Azotobacter *and* Clostridium butyricum. (*Depth of soil 2 cm.*)

State of Soil.	Inc. Days.	*Azotobacter* per gm.	Clostridia per gm.		pH.
			Total.	Spores.	
Initial	0	20	470	300	7·3
Moist, aerated, H_2O 6%. pF 3·9..	3	18	400	200	
	8	16	400	300	
	20	13	200	100	6·8
Wet, aerated, H_2O 16%. pF 2·2..	3	24	600	370	
	8	13	300	300	
	20	13	200	100	6·8
Saturated, H_2O 28%..	3	11	800	400	
	8	13	500	200	
	20	13	200	100	7·0
Waterlogged, H_2O 38%	3	20	1,600	330	
	8	20	200	200	
	20	13	300	200	7·0

The numbers of *Azotobacter* showed neither any appreciable increase after prolonged incubation, nor any decline as the soil moisture increased. The counts of *Cl. butyricum* were slightly more variable, and were generally higher in the waterlogged than in the drier samples; in all cases they outnumbered *Azotobacter*. In view of the increase in *Cl. butyricum* observed previously (Swaby, 1939), it would seem that anaerobic conditions were not obtained in the shallow plaques used. This is borne out by the occurrence of *Azotobacter* under all conditions and the fact that many of the clostridia exist as dormant spores.

A second experiment was carried out with the same soil brought to approximately the same pF values, but contained in jars 16 cm. in depth. Hereby the access of air to the bottom of the containers was prevented when the soil was waterlogged, but not when it was moist and crumbly. Otherwise the jars were treated as in the first experiment. The results are set out in Table 2.

TABLE 2.
Effect of Soil Moisture Content on Azotobacter *and* Clostridium butyricum. (*Depth of soil 15 cm.*)

State of Soil.	Inc. Days.	*Azotobacter* per gm.	Clostridia per gm.		pH.
			Total.	Spores.	
Initial	0	7	330	100	7·2
Moist, aerated, H_2O 7%. pF 3·8..	6	7	330	200	
	11	60	266	100	
	28	33	330	300	6·9
Wet, aerated, H_2O 16%. pF 2·2..	6	15	330	130	
	11	60	270	100	
	28	27	330	300	6·8
Saturated, H_2O 25%..	6	47	530	200	
	11	40	270	130	
	28	73	270	300	7·1
Waterlogged, H_2O 38%	6	7	1,000	330	
	11	22	200	140	
	28	33	330	300	7·2

The numbers of *Azotobacter* increased as incubation proceeded, but the numbers at different moisture content show no significant differences, although the numbers at 25% appear highest. The counts of *Cl. butyricum* show no significant increase after prolonged

incubation, and except for two fairly high values in saturated and waterlogged soil there is no obvious increase in their numbers as the pF values fall. The numbers of spores are still high in comparison with those of vegetative cells. The failure of the clostridia to respond to waterlogging and the slight development of *Azotobacter* under moist aerated conditions might possibly be due to the soil being deficient in organic matter available to the bacteria, especially as the sample was collected after a very dry summer. (The soil which previously showed increase in clostridia after waterlogging (Swaby, 1939) was collected in the spring and probably contained carbohydrates from plant residues.) A third experiment was therefore carried out, in which a small quantity of carbohydrate was added to the soil. Deep jars were filled with soil plus 0·2% glucose and made up to 4 different moisture conditions, and bacterial counts were made as before. The results are set out in Table 3.

TABLE 3.
Effect of Soil Moisture and Carbohydrate Content on Azotobacter *and* Clostridium butyricum.

State of Soil.	Inc. Days.	*Azotobacter* per gm.	Clostridia per gm.		pH.
			Total.	Spores.	
Initial	0	5	400	80	7·2
Moist, aerated, H_2O 8%. pF 3·7 ..	3	110	1,100	670	
	8	4,000	640	270	
	15	20,000	1,600	800	
	20	48,000	2,000	1,000	7·0
Wet, aerated, H_2O 14%. pF 2·8 ..	3	100	2,600	2,100	
	8	7,000	3,200	3,000	
	15	60,000	12,000	10,000	
	20	120,000	12,000	10,000	7·4
Saturated, H_2O 20%..	3	90	3,200	3,000	
	8	180	7,700	3,000	
	15	6,700	12,000	12,000	
	20	30,000	14,000	10,000	7·7
Waterlogged, H_2O 24%	3	4	3,100	1,400	
	8	67	7,700	3,000	
	15	2,000	16,000	18,000	
	20	1,000	13,000	14,000	7·2

Azotobacter, as might be expected, showed in all cases a rapid increase from a few to thousands per gm. of soil, but in the waterlogged soil the final numbers were lower and the rate of increase not so rapid. The counts of clostridia showed a similar increase as the incubation proceeded, and their final numbers were highest in the waterlogged soils which smelled of butyric acid; throughout the whole experiment the number of spores was large and quite the reverse of what might be expected. Generally the results are in full agreement with those of Winogradsky (1926). The high counts of *Azotobacter* under wet conditions might be due to surface growth, but the relatively strong increase in clostridia in the aerated soils is less readily explicable. Either the clostridia existed in association with protective aerobes, or else the soil must have contained unknown reducing substances capable of lowering the oxidation-reduction potential.

Under field conditions it is unlikely that the amount of soluble carbohydrate would ever amount to anything like 0·2% glucose. Therefore a fourth experiment was designed to imitate the conditions after the stubble of a four-ton-per-acre crop of oats had been ploughed in: 0·4% very finely ground oaten hay was added to University soil of two moisture conditions—moist aerated, and waterlogged. It was conceivable that the balance between aerobic and anaerobic nitrogen fixers might be influenced by the presence of an oxidizing agent which theoretically ought to favour *Azotobacter* and suppress the clostridia. The only important oxidizing substance naturally occurring in

the soil is manganese dioxide; therefore, 0·5% active MnO_2 was added to both aerated and waterlogged soil with oaten hay. The soils were incubated at 22°C. in 15 cm. deep jars, and bacterial counts made. Table 4 gives the results.

TABLE 4.

Influence of Hay and Manganese Dioxide on Azotobacter *and* Cl. butyricum *in Soil at Different Moisture Content.*

State of Soil.	Treatment.	Inc. Days.	*Azotobacter* per gm.	Clostridia per gm.	
				Total.	Spores.
Initial		0	15	500	400
Moist, aerated, H_2O 11%. pF 3·4.	Control	3	50	500	500
		6	50	500	400
		10	50	1,700	500
	0·4% hay	3	40	500	400
		6	100	700	300
		10	100	1,000	600
	0·4% hay, 0·5% MnO_2	3	40	500	400
		6	60	600	300
		10	75	1,200	800
Waterlogged, H_2O 20% ..	Control	3	20	600	400
		6	60	300	300
		10	60	1,500	800
	0·4% hay	3	600	1,000	400
		6	2,700	1,000	800
		10	14,400	3,200	1,200
	0·4% hay, 0·5% MnO_2	3	38	1,000	400
		6	66	1,000	1,000
		10	100	2,000	2,000

Both under aerated and waterlogged conditions there was a progressive increase in *Azotobacter* and *Cl. butyricum*, and especially in the jars with hay. At 11% moisture the addition of hay increases the numbers of *Azotobacter* and clostridia only slightly, and MnO_2 has no effect on either group of organisms. Under waterlogged conditions the results were quite different: the growth of *Azotobacter* is strongly stimulated by the hay, but suppressed by extra addition of MnO_2; the growth of clostridia is but slightly stimulated by the hay and not retarded by MnO_2. It would seem that MnO_2 was reduced under waterlogged conditions, and that toxic concentrations of manganese ions have inhibited the growth of *Azotobacter*, whereas on the other hand it is possible that the obligate anaerobe by adaptation has become less sensitive to such ions.

The stronger growth of *Azotobacter* in waterlogged soil agrees fully with what was found in other experiments with straw (Jensen, 1940). Two days after conclusion of the above experiment, counts were carried out from the top and bottom layers of soil with 20% moisture and 0·4% hay. Result:

	Azotobacter per gm.	Clostridia per gm.	
		Total.	Spores.
Top	100,000	5,000	4,000
Bottom	0	5,000	3,000

As might be expected, *Azotobacter* grows only on the surface of the waterlogged soil, while the clostridia seem to live in symbiosis with aerobes in the upper layers and to exist as normal anaerobes in the lower levels.

These experiments show clearly that, in the absence of an available food supply, the clostridia as well as *Azotobacter* remain largely dormant and are little influenced by the air-water relationship of the soil. In the presence of glucose or hay both groups multiply rapidly, the clostridia being especially favoured by waterlogged conditions and therefore presumably of most significance as nitrogen fixers in periods after heavy rainfalls. Being more resistant to acid reaction than *Azotobacter*, they might be of significance in this respect in many soils unsuitable for the former organism. An experiment was therefore designed to test whether the multiplication in soil is accompanied by a measurable nitrogen fixation, as a supplement to a previous series of experiments (Jensen, 1940) in which systematic counts of clostridia were not carried out, but where the conditions were usually favourable for *Azotobacter*, and where gains of nitrogen were always bound up with vigorous growth of this organism.

2. *Numbers of Clostridia and Azotobacter in Relation to Nitrogen Content of Soil.*

The soil used for this experiment was a typical wheat soil: light red loam of moderately acid reaction (pH 5·5), from Condobolin Experiment Farm, N.S.W. Before the experiment it was washed free from nitrate, air-dried, and ground very finely. The soil was used both at its original acid reaction and at a faintly alkaline reaction produced by addition of lime. Four portions of 900 gm. air-dry soil were given additions of 1·0% finely ground wheat straw, 10% of a solution containing 0·2% KH_2PO_4 and 0·005% Na_2MoO_4, and water to complete saturation (31% moisture); the water included 10 c.c. of a suspension of a soil rich in *Azotobacter* and clostridia. Two of the portions received further 0·25% $CaCO_3$. The carefully mixed portions were placed in loosely-covered glass jars where they formed a layer 15 cm. deep. The jars were incubated at 24–27°C. for 14 weeks, and samples were drawn at intervals for bacterial counts and chemical analysis. Nitrogen was determined as previously (Jensen, 1940), except that reduced iron was used instead of zinc for reduction of nitrate which, however, could hardly be detected; selenium was used as a catalyst. Organic carbon was determined by the method of Walkley and Black (1934). Owing to the high price of pyrogallic acid under the war conditions, the counts of clostridia were, after the third week, made by the dilution method in glucose solution, and are only very roughly approximate. The results are given in Table 5 (figures for carbon and nitrogen

TABLE 5.

Numbers of N-fixing bacteria and chemical changes in soil+straw.

A. Numbers of bacteria.

Inc. Days.	$-CaCO_3$			$+CaCO_3$		
	Azotobacter per gm.	Clostridia, 1,000 per gm.		*Azotobacter* per gm.	Clostridia, 1,000 per gm.	
		Total.	Spores.		Total.	Spores.
Initial	<2	0·020		<2	0·020	
7 d. *a*	(0)	6–16	16–32	11,300	3·6–8	8–32
b	(0)	6–24	8–24	1,630	6–12	1·6–3·2
14 d. *a*	1,310	6	2	6,860	4	2
21 d. *b*	60	15–20	2–4	>60,000	6–7	3–4
28 d. *a*	<15	<10		24,100	<10	
b	<15	<10		19,300	10–50	
60 d. *a*	<8	<1		39,000	>5	
b	180	<1		63,100	>5	
98 d. *a*	4	<1		30,000	>5	
b	4	<1		24,700	>5	

TABLE 5.—*Continued.*

B. Chemical changes. (Average of duplicate jars except $-CaCO_3$, 98 d.)

Series and time of incubation.	n*	Total N, p.p.m.	Gain or loss of N, p.p.m.	Organic C, %	Loss of C, %	pH.
Original	10	728±3·5		1·36		5·5
$-CaCO_3$, inc. 28 d.	8	727±3·6	−1±5·0	1·25	0·11	6·0–6·1
Do. 60 d. ..	9	727±2·6	−1±4·4	1·25	0·11	6·0
Do. 98 d. *a* ..	3	689±3·8	*−39±5·1*	1·25	0·11	6·2
b ..	5	728±5·8	0±6·8	1·23	0·13	6·2
$+CaCO_3$, 28 d. ..	8	734±1·9	+6±4·0	1·21	0·15	7·1–7·2
Do. 60 d. ..	8	724±2·9	−4±4·6	1·22	0·14	7·1
Do. 98 d. ..	7	726±4·8	−2±5·9	1·18	0·18	7·3–7·4

* Number of parallel determinations of N.

are the means of duplicate jars* which agreed completely except in one case: $-CaCO_3$, 98 days, where the separate values for each jar are given).

As was to be expected, *Azotobacter* developed vigorously in the limed soil, but only scantily in the unlimed soil which became slightly less acid during incubation. The clostridia multiplied rapidly at both reactions. The chemical data show absolutely no gain of nitrogen, even in the limed soil (the small apparent gain after 4 weeks is well below significance); it is noteworthy that *Azotobacter* is represented by numbers far lower than in previous experiments where fixation could be detected (Jensen, 1940). The only significant change is an appreciable loss of nitrogen in one jar without lime after 98 days. The loss of carbon is considerable, especially in the limed soil, and is most rapid during the first 4 weeks.

It seems clear that no considerable gains of nitrogen can be expected through the activity of clostridia in soil after heavy rains, even if, as in the present case, (*a*) nitrate is absent, (*b*) undecomposed plant residues are present, and (*c*) vigorous multiplication of the clostridia takes place, accompanied by rapid decomposition of the plant residues (in the present instance the losses of carbon during the first 4 weeks correspond to some 25–35% of all the carbon introduced with the straw).

3. *Further Observations on the Distributions of Azotobacter.*

The summer season 1938–39 was remarkable for a severe drought accompanied by an exceptional heat wave in January 1939. This afforded an opportunity of testing whether *Azotobacter* becomes particularly active after a period of dry heat (cf. Wilsdon and Ali, 1922). From the breaking of the drought (March 1939) to the early summer (November 1939) 52 soil samples were examined for the presence of *Azotobacter* by the same methods as previously (Jensen, 1940). The soils were all from the wheat belt of New South Wales, except 2 from South Australia. Another 6 samples were examined in May 1940. *Azotobacter* was found in 10 soils only, as shown below:

* The table gives the mean values and the standard error calculated according to the formula: $m = \sqrt{\frac{S(x-\bar{x})^2}{n(n-1)}}$, where $S(x-\bar{x})^2$ represents the sum of squares of deviations from the mean, and n the number of parallel determinations. Standard error of the difference: $\sqrt{m_1^2 + m_2^2}$.

Soil.	pH.	*Azotobacter.* Pellicle in mannite solution.	*Azotobacter.* Plate Count per gm.
Red loam, Forbes	5·8	+	3
Red loam, Manilla	6·0	+	21
Light brown loam, Dirnaseer	6·3	+	0
Light grey loam, Tamworth	6·3	+	1
Brown loam, Tamworth	6·6	+	1
Grey sandy loam, Cowra	6·7	+	31
Heavy brown loam, Griffith	7·7	+	0
Heavy grey loam, Narrandera	7·7	−	3
Limestone soil, Tamworth	7·7	+	48
Heavy brown loam, Griffith	8·5	+	600

Azotobacter is thus by no means more frequent than in the previous series of investigations. Most of the samples contained fresh roots of leguminous plants, especially *Medicago denticulata* and *Trifolium glomeratum.* Beijerinck (1909) referred to frequent occurrence of *Azotobacter* in the rhizosphere of legumes; this could not be corroborated from the present observations. A general survey of the distribution of *Azotobacter* in relation to soil reaction is given in Table 6, which is based on 85 data from previous observations (Jensen, 1940), the present 58, and 80 data on Victorian soils by Swaby (1939) who used a very similar method. The results depart in no way from what has been found in other parts of the world.

TABLE 6.
General Distribution of Azotobacter *in Relation to Soil Reaction.*

pH-Range.	Number of soil samples. Jensen (1940). Total.	Az.+.	Swaby (1939). Total.	Az.+.	Combined data. Total.	Az.+.	Az.+ %.
4·0–5·0	5	0	9	1	14	1	(7)
5·1–6·0	69	7	35	4	114	11	9·6±2·76
6·1–7·0	47	15	18	6	65	21	32·3±5·80
7·1–8·0	19	14	15	8	34	22	64·7±8·18
8·1–9·0	3	1	3	2	6	3	(50)
Total	143	37	80	21	223	58	26·0±2·94

Shortly after the beginning of this series of investigations there appeared a paper by Starkey and De (1939) on *Azotobacter indicum*, which is resistant to acidity, sensitive to calcium carbonate, and incapable of utilizing dextrine. Since *Az. indicum* might be of some significance in the nitrogen economy of acid soils, and since it could evidently not have been detected by the methods hitherto used, a special search was made for it in 50 of the above-mentioned soils, of pH 4·6 to 8·5, including 6 irrigated rice soils. Triplicate plate counts in a dilution 1:10 were made on agar corresponding to the saccharose solution of Starkey and De (1939), with incubation 3 weeks at 28–30°C. In no case was *Az. indicum* found, but two authentic strains (kindly supplied by Dr. R. L. Starkey, New Jersey Agr. Exp. Station, U.S.A.) grew well on the medium and produced characteristic colonies thereon when added to soil suspension. The method is thus adequate, and it seems that *Az. indicum* does not occur at all in Australian soils, or else it must be so rare as to be without any practical significance.

Several plates of saccharose-agar showed big slimy colonies of a polysaccharide-forming yeast; 3 strains of this and 6 strains of bacteria of the type studied by Selim (1931) were tested for nitrogen-fixing power. The bacteria were isolated from wheat

soils by inoculation into soil extract with glucose, and plating on corresponding agar; two of them were similar to the "bacille gommeux" of Winogradsky (1926), one apparently identical with *Bac. malabarensis* Löhnis and Pillai (1907), and 3 were gas-producing rods of the *Bact. aerogenes*-type. Tests for nitrogen fixation were done by cultivation for 20 to 30 days at 28–30°C. in (*a*) Starkey and De's saccharose agar, (*b*) corresponding solution with 2% soil (cf. Selim, 1931), and (*c*) NO_3-free soil extract with 2% glucose and mineral nutrients including molybdenum. *Azotobacter* and *Rhizobium trifolii* were grown as controls. Neither the tested organisms nor the rhizobia showed nitrogen fixation; the change in N-content of cultures before and after incubation never exceeded 0·13 mgm. under conditions where *Azotobacter* fixed from 1 to 7·2 mgm. N per culture, or up to 14 mgm. per gm. of sugar supplied. In view of these and previous results (Jensen, 1940) we may safely conclude that no further attention need be given to the possibility of gains of nitrogen in soil through the agency of non-symbiotic microorganisms other than *Azotobacter*, clostridia, and blue-green algae.

Summary.

Waterlogged soil conditions and presence of decomposible organic matter (glucose or oaten hay) were found to promote the development of *Clostridium butyricum*, which also occurred in fairly large numbers in aerated soil, presumably by existing in association with aerobes. In the absence of glucose or hay, clostridia as well as *Azotobacter* remained largely dormant without being much influenced by the air-water relationship of the soil. Manganese dioxide depressed the numbers of *Azotobacter* in saturated soil with hay, but had no effect on the clostridia, or on *Azotobacter* in aerated soil.

Nitrogen fixation could not be detected in acid or neutral, water-saturated soil incubated with addition of wheat straw, in spite of good multiplication of the clostridia at both reactions, and vigorous decomposition of the organic matter of the straw. No appreciable gains of nitrogen in the soil through the activity of clostridia can therefore be expected in periods during and after heavy rains.

No evidence was found that *Azotobacter* is prominent in soil after droughts, or in the rhizosphere of leguminous plants. The acid-resistant *Az. indicum* was not found in Australian soils, nor any other aerobic organism capable of non-symbiotic nitrogen fixation.

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